



Screening and HPLC quantitation of phytochemicals with antiobesity activity in traditional teas consumed in Zimbabwe

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ABSTRACT

Obesity is now a major problem, leading to life threatening complications such as hypertension and diabetes type II. This study was focused on screening and quantifying phytochemicals portraying α -amylase inhibitory activity in selected traditional herbal teas for possible use as ethnobotanical therapeutics for management of obesity. Phytochemicals were extracted using liquid-solid and solid phase extraction using C_{18} cartridges followed by a separating stage using Thin Liquid Chromatography. Quantitation of antiobesity phytochemicals was achieved using reversed phase HPLC. The three herbal teas that is *Myrothamnus flabellifolius*, *Lippia javanica* and *Ficus sur* were all found to consist of flavonoids. In addition *L. javanica* extract consisted of alkaloids, saponins and terpenoids while *M. flabellifolius* extract consisted of saponins and alkaloids. *F. sur* also consisted of alkaloids. Flavonoids from all the three plants showed antiobesity activity while alkaloids failed to inhibit α -amylase. *M. flabellifolius* flavonoids exhibited the highest percentage inhibition of α -amylase activity of 88 %, followed by *L. javanica* at 83 % and lastly *F. sur* at 81 %. The concentration of flavonoids in *M. flabellifolius* was found to be 256.92 ± 4.41 mg/mL while in *L. javanica* was 125.60 ± 2.16 mg/mL. *F. sur* exhibited the lowest flavonoid content of 118.61 ± 2.04 mg/mL. The present research shows that dietary flavonoids from the three herbal teas can be used as ethnobotanical therapeutics for management of obesity.

Key words: *Myrothamnus flabellifolius*, *Lippia javanica* and *Ficus sur*, obesity, phytochemicals

INTRODUCTION

Obesity is now a serious disease whose negative effects on the human population have become a global concern. Obesity is the over-accumulation of fat in the body resulting mainly from a higher than required intake of foods consisting of large amounts of carbohydrates, or simply the intake of energy exceeding its expenditure [1]. Excessive carbohydrates in the diet is converted to fats. Being obese is more than just being overweight. An obese individual has a body mass index (BMI) exceeding 30 [2]. BMI index is the ratio of body mass to height. The excessive body fat of an obese person predisposes him or her to develop chronic non-communicable health problems such as stroke, type II diabetes, hypertension and various cancers such as colon, breast, endometrium and kidney cancers [1]. If one is obese, one is susceptible to psychological effects such as depression, low self-esteem, anxiety and obsessive behaviour [3]. Men who are obese have increased risk of developing impotence and infertility while females may suffer from hormonal imbalance which may lead to infertility [4]. Obesity incidences are higher in developed nations like the USA but trends also show a progressive rise of cases in developing nations and China which used to have low rates [5]. Future projections show a continual increase of obesity worldwide [6]. In order to

reduce the contributions of obesity to social dysfunction, morbidity and mortality, individuals and nations have incurred huge costs in health care [6].

Efforts to reduce obesity by encouraging physical activity resulted in limited success, as shown by the upward trend in numbers of obese people worldwide. Research in anti-obesity drug development has produced several drugs showing considerable efficacy in obesity reduction, notably orlistat and sibutramine [7]. These drugs however have been shown to have negative side effects and their continued use beyond one year is not advised [7]. There is need therefore to develop alternative methods and drugs to combat obesity and natural products have shown potential as a source of compounds with obesity reducing ability [8]. The reduction of fat and carbohydrate absorption has been shown to reduce obesity since excess fat and carbohydrates are used to build and deposit new fat tissue in the body [1]. Orlistat itself acts by the inhibition of pancreatic lipase, the principal fat digesting enzyme in the duodenum and α amylase responsible for carbohydrate metabolism [1] while sibutramine works by reducing the appetite for food [9].

Studies have shown the presence of pancreatic lipase and α -amylase inhibiting compounds in the tea plant *Camellia sinensis* leaves [10]. The compounds in tea include polyphenols, flavonoids and saponins [11]. The catechin, epigallocatechin gallate (EGCG) has a high potency in both pancreatic lipase and amylase inhibition [12]. *Myrothamnus flabellifolius*, *Lippia javanica* and *Ficus sur* are widely distributed plants traditionally used as herbal teas in Zimbabwe which make them interesting candidates of study as possible sources of antiobesity phytochemicals [13]. *Lippia javanica* (Zumbani), was shown to contain flavonoids and essential oils [14]. *Ficus sur* (Muonde), showed the presence of polyphenols and alkaloids [15]. *Myrothamnus flabellifolius* (Mufandichimuka) was shown to contain alkaloids, flavonoids and saponins [16]. Because some polyphenols and flavonoids have been demonstrated to have enzyme inhibition and therefore anti-obesity activity, these traditional green teas may potentially have anti-obesity activity. Apparently no study has been done to link these traditional green teas to anti-obesity activity. Thus this study was designed to isolate, screen and quantify phytochemicals that have anti-obesity activity from the herbal tea extracts.

MATERIALS AND METHODS

2.1 Equipment

A Mettler Toledo digital analytical balance AB204-S was used to measure the masses of samples during sample preparation and reagents during the preparation of solutions. A Labotec horizontal shaker was used to agitate the solvent-powder mixture during liquid-solid extraction so as to optimize the extraction. A KnF Neuberger vacuum suction pump was used to enhance filtration to separate the liquid sample from the solid residue during sample preparation. The Jenway 3510 pH meter was used to adjust pH during the preparation of the phosphate buffer. The Genesys 10s UV/Vis Spectrophotometer was used to determine percentage inhibition of α -amylase by the phytochemicals. The Varian HPLC UV Prostar 325 system was used in the quantitation of phytochemicals exhibiting antiobesity activity.

2.2 Chemicals

HPLC grade solvents were used in all HPLC procedures. All other reagents were analytical reagent grade.

2.3 Sample collection and pre-treatment

Lippia javanica, *Myrothamnus flabellifolius* and *Ficus sur* leaves were collected fresh around the St Pauls Musami area in Murewa on 12 March 2015. The plants were authenticated by a research officer at The National Herbarium and Botanic Garden in Harare. The samples were then shade dried. The dried leaves of each of the three plants were ground to a powder using a wooden pestle and mortar and passed through a 0.75 μ m sieve. The resulting fine powders were packed and stored in polythene bags in a cupboard prior to liquid-solid extraction.

2.4 Sample preparation

2.4.1 Liquid-Solid Extraction

Powdered sample (10g) of the *Lippia javanica* leaves was weighed and mixed with 100mL of analytical grade absolute ethanol in a 250mL conical flask. Another 10g were mixed with ethyl acetate. The samples were shaken for 30 minutes on a horizontal shaker. The samples were then filtered using Whatman's grade no. 1 filter paper and placed in reagent bottles. The procedure was repeated for the other two samples.

2.4.2 Solid Phase Extraction (SPE)

This SPE was performed with minor modifications of the previous procedure [17]. Each SPE cartridge was conditioned by passing 5mL of absolute ethanol through 0.4g of the Hydrophobic-Lipophilic Balanced (HLB) sorbent by gravity in an SPE cartridge. Equilibration was achieved by passing 5mL of distilled water through the sorbent. This was followed by loading the extract through the cartridge again under gravity. To maximize the sample clean-up 1 mL of acetone was used to wash the sorbent. The final stage was elution using 5mL of absolute ethanol. The samples were stored in glass vials. The process was repeated for the ethyl acetate extracts by replacing ethanol with ethyl acetate.

2.5 Analytical Thin Liquid Chromatography (TLC)

A previous TLC method [18] was used and modified as follows; 10 x 10 cm aluminium silica gel coated TLC plates were cut to size 10.0 x 1.5cm and activated by heating at 100°C for 10 minutes before being allowed to cool to room temperature. A pencil line was drawn 1.5cm from one edge. The sample was spotted with a thin capillary pipette onto the pencil line. The plate was placed in a developing chamber with a solvent. The development chamber was a 250ml beaker closed with a taped plastic sheet. The following solvent systems were tested in order to determine the best mobile phase that could separate the phytochemicals efficiently; chloroform: ethyl acetate: formic acid (5:4:1 v/v/v), acetic acid: chloroform (1:9), petroleum ether: ethyl acetate (7:3), acetone: hexane (1:3). Of these only the petroleum ether: ethyl acetate system showed clear separation and was used in all samples. The following spraying agents were applied: (1) Mayer's reagent (testing for alkaloids) – made by dissolving 1.36g mercuric chloride and 5.0g potassium iodide in 100mL water; (2) 10% ethanolic sulphuric acid (terpenoids) – made by dissolving 10ml concentrated sulphuric acid in 100mL absolute ethanol; (3) 5% aluminium chloride in distilled water (flavonoids) and (4) Saturated iodine chamber (saponins) – made by warming a few iodine grains in a dry closed 250mL conical flask with the developed TLC plate inside.

2.6 Preparative TLC

Pre-coated thick silica gel on glass TLC plates were used. Each plate was 20 x 20cm. The solvent used was the petroleum ether: ethyl acetate (7:3) mixture. Each of the ethanol and ethyl acetate extracts was streaked 1.5cm from the edge of its respective TLC plate and allowed to dry. When the sample was dry the plate was gently placed in the development tank and allowed to develop. When the solvent front had moved up to 15cm the plate was removed and the position of the solvent front marked immediately with a pencil. The R_f values of the different bands were then computed. The bands corresponding to those that tested positive for the phytochemicals (flavonoids, alkaloids and saponins) in the analytical TLC, were each scratched off. The scratched material from each band was mixed with 5mL of absolute ethanol allowed to stand for 10 minutes and then filtered with Whatman's no. 1 filter paper and then placed in a glass vial.

2.7 Alpha amylase inhibition test

Preparation of phosphate buffer solution

A phosphate buffer solution of pH 6.9 was prepared by weighing 0.86g of potassium dihydrogen phosphate dehydrate ($\text{KH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) and 1.79g of disodium hydrogen phosphate 12-hydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) and dissolving the mixture in 1000mL of distilled water at 25°C with the aid of a pH meter the pH was adjusted to the required 6.9 by adding a little phosphoric acid and sodium hydroxide.

Preparation of enzyme solution

Alpha amylase (0.5g/L) was prepared by dissolving 0.5g of the enzyme in 1000mL of distilled water at 30°C. 250µL of extract and 250µL of phosphate buffer containing alpha amylase were injected into a test tube and pre-incubated for 10 minutes at 30°C. 250µL of 1% starch in phosphate buffer was injected into the mixture and this was incubated for 10 minutes at 30°C. Termination was by adding 100µL of 0.1 M hydrochloric acid and heating at 100°C for a further 5 minutes. 100µL of 1% iodine solution was then injected into the cooled mixture. Absorbance was tested at 546nm against a control of similar constitution but without the extract. This was the negative control with no inhibitor. Percentage inhibition was calculated using the equation:

$$\% \text{ inhibition} = \frac{Ac - As}{Ac}$$

Where A_c is absorbance of control and A_s is absorbance of sample. A positive control of 0.5% mercuric chloride was also used and arbitrarily assigned a percentage inhibition of 100%. This was a modification of the anti-obesity tests reported previously [19], [20].

2.8. HPLC Analysis

2.8.1 Mobile phase preparation

The mobile phase was made by adding acetic acid (10mL) to 1000mL of HPLC grade water in a volumetric flask. 750mL of this mixture was added to 250ml methanol and the mixture was degassed in a sonicator at 30°C for 5 minutes.

2.8.2 Standards preparation

A 0.30 g/L stock solution was made by dissolving 0.30g of catechin in 1000ml of HPLC grade absolute ethanol. Serial dilution was used to produce a series of lower concentrations. Catechin standard solutions 10, 20, 100, 150 and 300mg/mL were prepared by serial dilution of the stock solution. Using these concentrations and the peak areas obtained from HPLC a calibration curve was made. Concentrations of flavonoids in the plant extracts were determined using the calibration method.

2.8.3 Chromatographic analysis

This was done on a Varian HPLC equipped with a variable UV Prostar 325 detector. The sample was manually injected using a Rodyne injector. The injection volume used was 20 μ L and a flow rate of 1 mL/min was employed. The detector operated under the VarianStar/Galaxie Chromatography Workstation software version 6. The HPLC column used was the Varian Microsorb MV 1005 250x4.6mm id, packed with 5 μ m particles of C18 stationary phase. Separation was in the isocratic mode. The detector was set at 360nm obtained from a prior scan of sample on a UV spectrophotometer (Genesys 10sUV-Vis). This was a modification of a method reported previously [17] and was used to first analyze the standard and then the samples.

RESULTS AND DISCUSSION

3.1 Analytical TLC

Results for the analytical TLC are shown in Fig 1 and Table 1. Table 1 shows retention factors of four spots (at least four types of compounds) separated from the ethanol extract of *M. flabellifolius*, six from *L. javanica* and three from *F. sur.* Fig 1 shows that the phytochemicals were separated efficiently using the mobile phase, petroleum ether and ethyl acetate in the ratio 7:3. The petroleum ether: ethyl acetate solvent was also found to be the best mobile phase for separating polyphenols in *Camellia sinensis* [21].

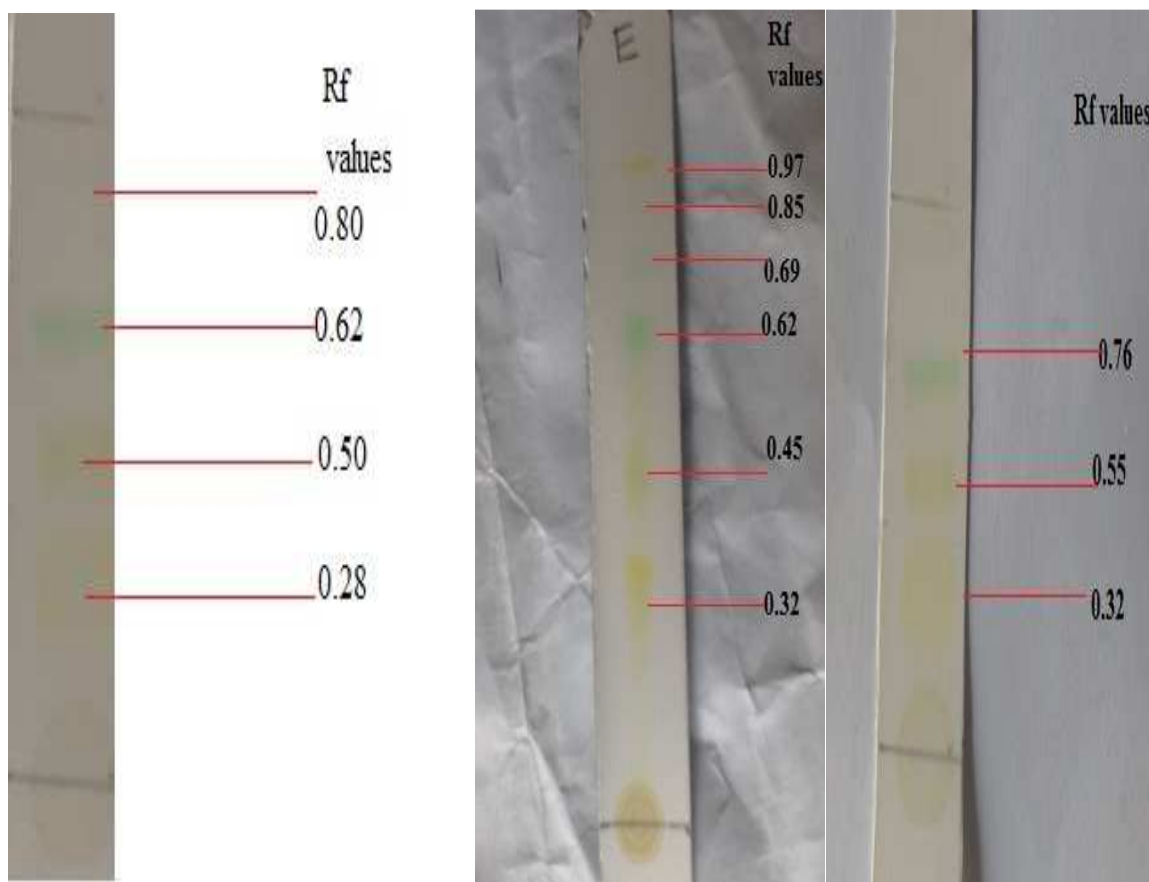


Fig 1 ethanol extract of *M. flabellifolius* showing four spots, *L. javanica* showing six spots, *F. sur* showing three spots and their R_f values

Table 1 R_f values of compounds for ethanol extracts

Plant	Number of spots	R_f values
<i>M. flabellifolius</i>	4	0.28
		0.50
		0.62
		0.80
<i>L. javanica</i>	6	0.32
		0.45
		0.62
		0.69
		0.85
		0.97
<i>F. sur</i>	3	0.33
		0.55
		0.97

Table 2 shows four spots from the ethyl acetate extract of *M. flabellifolius*, five from *L. javanica* and three from *F. sur*. The last column shows the calculated retention factor values.

Table 2 R_f values for compounds from ethyl acetate extracts

Plant	Number of spots	R_f values
<i>M. flabellifolius</i>	4	0.32
		0.52
		0.63
		0.71
		0.19
		0.31

<i>L. javanica</i>	5	0.44
		0.73
		0.94
		0.36
		0.55
<i>F. sur</i>	3	0.55
		0.71

3.2 Phytocompound identification

Table 3 shows that the herbal teas consist of different types of phytochemicals. All the teas contain flavonoids. *M. flabellifolius* also contains alkaloids and saponins while *L. javanica* contains alkaloids, saponins and terpenoids. *F. sur* also consists of alkaloids. The results show that ethanol was a superior solvent for extracting phytochemicals than ethyl acetate therefore ethanol extracts were kept for further analysis.

Table 3 Summary of tests for flavonoids, saponins, alkaloids and terpenoids

	<i>M. flabellifolius</i>		<i>L. javanica</i>		<i>F. sur</i>	
	Ethanol	Ethyl acetate	Ethanol	Ethyl acetate	Ethanol	Ethyl acetate
Alkaloids	++	-	+	+	+	++
Flavonoids	++	+	+	+	++	+
Saponins	+	-	+	+	-	-
Terpenoids	-	-	+	-	-	-

- Phytocompound test negative
 + Phytocompound test positive
 ++ Phytocompound test (colour more intense)

3.3 Preparative TLC Analysis

Fig 2 shows the chromatogram from the development of the TLC plate after spotting the ethanol extract of *M. flabellifolius*. The bands: $R_f = 0.28$ (positive for flavonoids), $R_f = 0.62$ (positive for saponins) and $R_f = 0.80$ (positive for alkaloids) were scratched, dissolved in ethanol and the filtrate kept for enzyme inhibition tests and HPLC analysis.

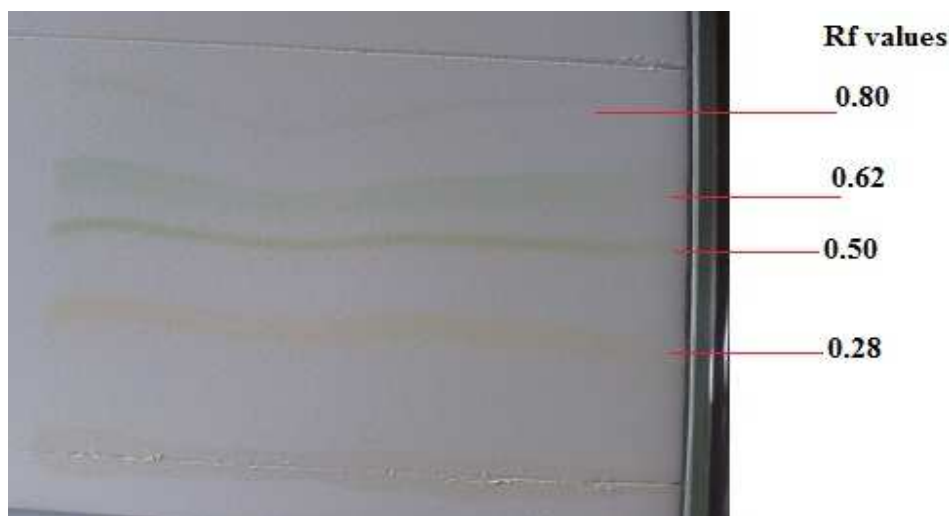


Fig 2 Preparative TLC chromatogram of *M. flabellifolius*

Fig 3 shows the chromatogram for the ethanol extract of *L. javanica*. The bands 0.32, 0.62, and 0.85 positive for flavonoids and band 0.97 positive for alkaloids were scratched for enzyme inhibition tests.

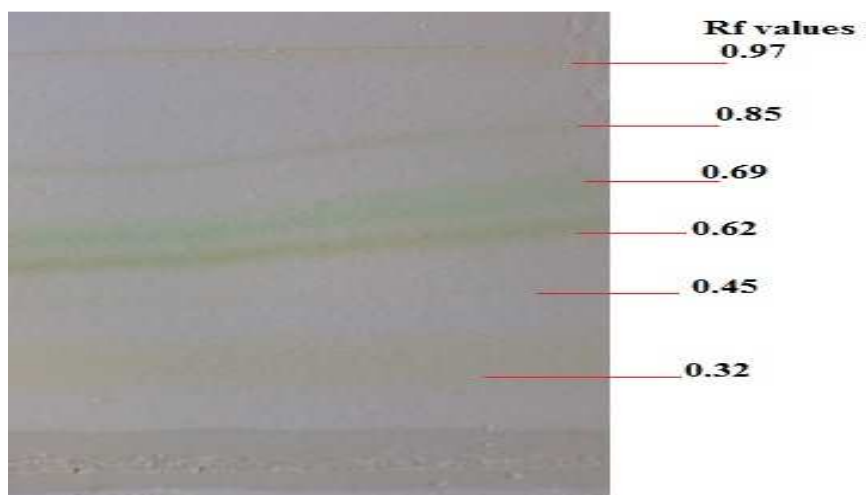


Fig 3 Preparative TLC chromatogram of *L. javanica*

Fig 4 shows the chromatogram for the ethanol extract of *F. sur* from which bands 0.32 and 0.76 consisting of flavonoids were scratched and used for enzyme inhibition tests.

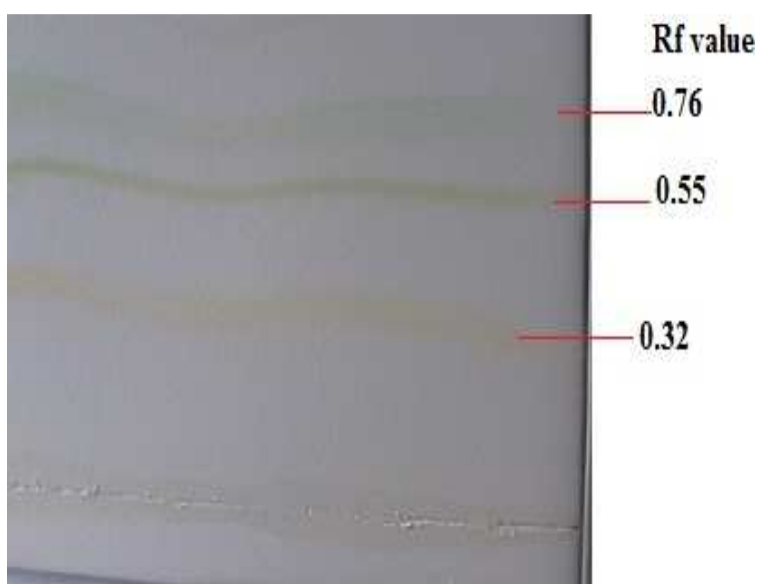


Fig 4 Preparative TLC chromatogram of *F. sur*

3.4 α -amylase inhibition test

Table 4 and Fig 5 show results for inhibition of α -amylase activity. Fig 5 shows extracts with the highest α -amylase inhibition and two controls: (a) negative control with no inhibitor and therefore maximum digestion of the starch, (b) mercuric chloride control showing the blue of undigested starch at 100% inhibition (c) *M. flabellifolius* extract from preparative TLC band 1 ($R_f = 0.28$) showing the blue representing inhibited enzyme activity, (d) *L. javanica* from band 3 ($R_f = 0.62$) shows inhibited enzyme action, (e) *F. sur* from band 1 ($R_f = 0.32$) shows a lighter colour to indicate lower inhibition. The results shows that flavonoids isolated from all the three plants inhibits α -amylase activity while no inhibitory activity was observed for alkaloids. Table 4 shows that *M. flabellifolius* had the highest percentage inhibition at 88%, followed by *L. javanica* at 83% with *F. sur* third at 81%. A second spot on *L. javanica* had a lower percentage inhibition of 33%. Narkhede [19], reported percentage inhibitory activity of a reference standard acarbose of 71.52% which is significantly lower than the inhibitory values obtained for the three herbal teas.

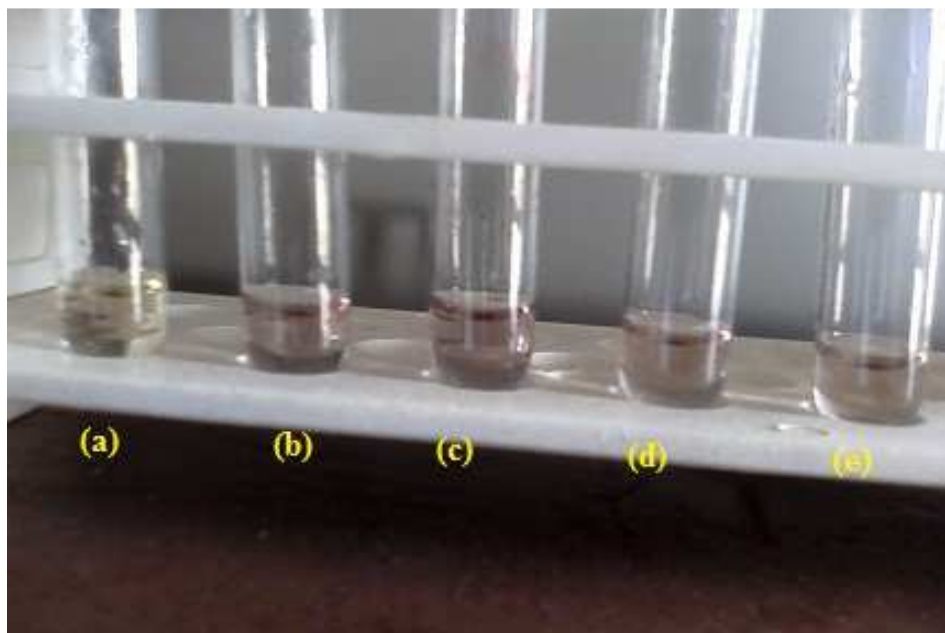


Fig 5. Qualitative comparison of α -amylase inhibition

Table 4 Percentage inhibition of phytochemicals on α -amylase activity

Plant	R _f value	Phytochemical	Percentage inhibition
<i>M. flabellifolius</i>	0.28	Flavonoids	88
	0.62	Saponins	0
	0.80	Alkaloids	0
<i>L. javanica</i>	0.32	Flavonoids	33
	0.62	Flavonoids	83
	0.85	Flavonoids	0
	0.97	Alkaloids	0
<i>F. sur</i>	0.32	Flavonoids	81
	0.76	Flavonoids	0

3.5 HPLC analysis

Fig 6 (a) and (b) below show the chromatograms obtained from the standard and one sample. Fig 6 (a) shows a catechin standard peak and (b) shows a peak of ethanolic *L. javanica* extract of R_f value 0.62. Catechin is eluted at a retention time of 2.91 minutes while the catechin equivalent in the *L. javanica*, is eluted at 3.02 minutes.

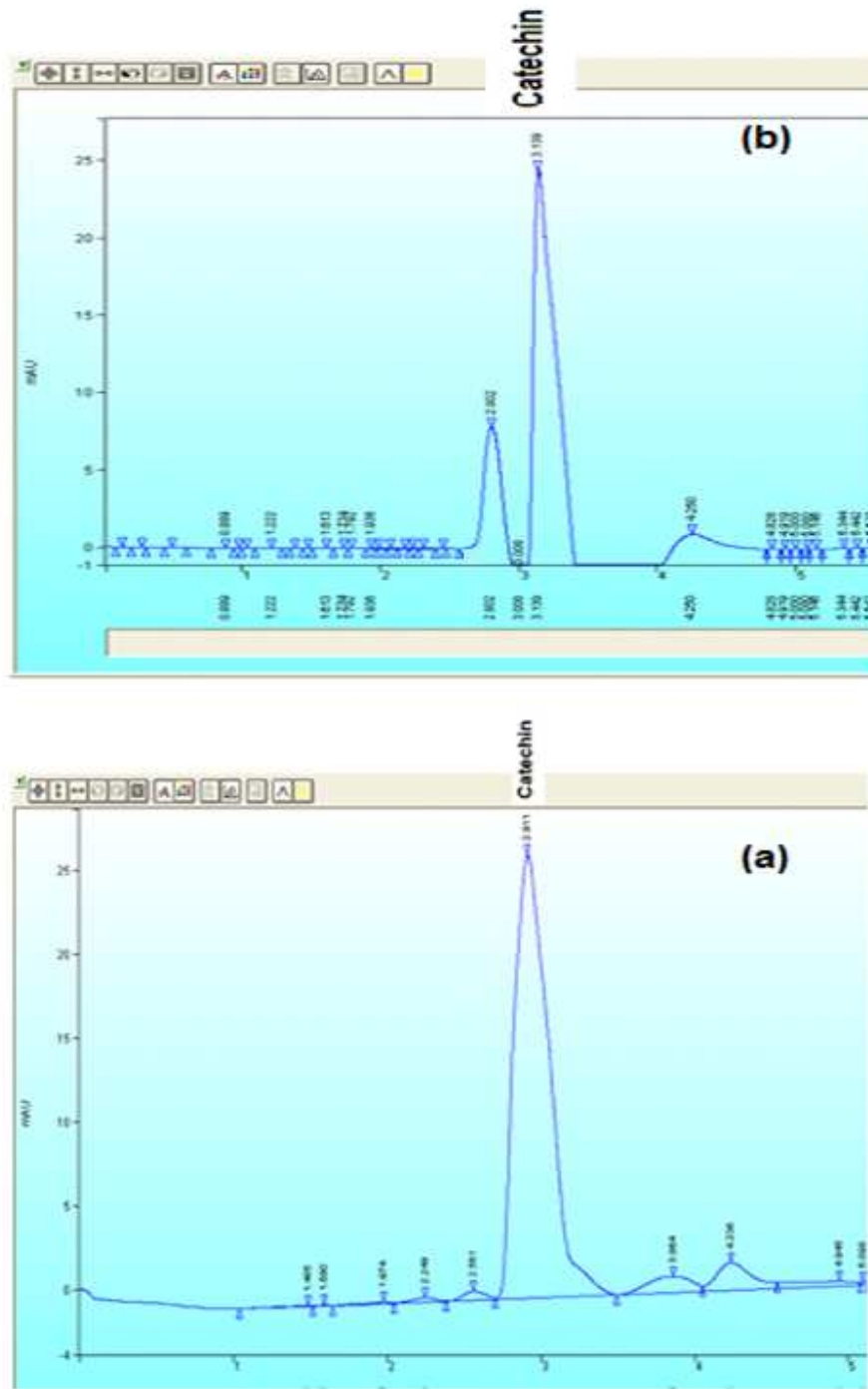


Fig. 6 HPLC chromatograms: (a) catechin standard and (b) ethanolic *L. javanica* extract from band with R_f value 0.62

Fig 7 shows the calibration curve obtained when peak areas from the HPLC runs of the catechin standard were plotted against concentration. A linear dynamic range of 10-300 mg/mL is shown. The value of the coefficient of variation shows that there was good correlation

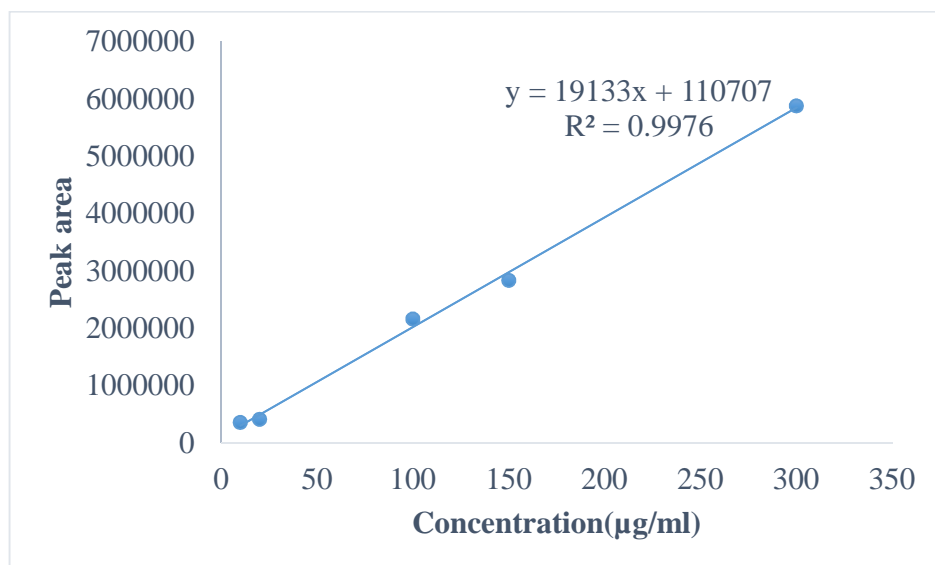


Fig 7 Calibration curve of a catechin standard

The phytochemicals from the extracts that tested positive for enzyme inhibition were quantified using the catechin calibration curve. Table 5 shows the concentrations per catechin equivalent of the three herbal teas

Table 5 Concentration of flavonoids in the plant extracts

Plant	R _f value	Concentration in mg/mL catechin equivalent
<i>M. flabellifolius</i>	0.28	256.92 ± 4.41
<i>L. javanica</i>	0.62	125.60 ± 2.16
<i>F. sur</i>	0.32	118.61 ± 2.04

CONCLUSION

Some phytochemicals namely flavonoids, saponins, alkaloids and terpenoids were all found in *L. javanica* in different concentrations. *M. flabellifolius* was found to contain flavonoids, alkaloids and saponins but no terpenoids. *F. sur* was found to contain alkaloids and flavonoids but no saponins or terpenoids. All extracts that tested positive for flavonoids showed alpha amylase inhibition and therefore anti-obesity activity. Alkaloids tested negative for alpha amylase inhibition. *M. flabellifolius* had the highest concentration of flavonoids which was significantly higher than that in both *L. javanica* and *F. sur*. *F. sur* had the least concentration of flavonoids. The results of this study showed that flavonoids present in the teas have anti-obesity activity therefore they can be used as ethnobotanical therapies for management of obesity and future sources of antiobesity drugs.

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